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NO. 1326

PROPHYLAXIS AND TREATMENT OF INFLUENZA A VIRUS INFECTION BY CARRIER-MEDIATED PASSIVE IMMUNITY

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J.P. Wong and L.L. Stadnyk

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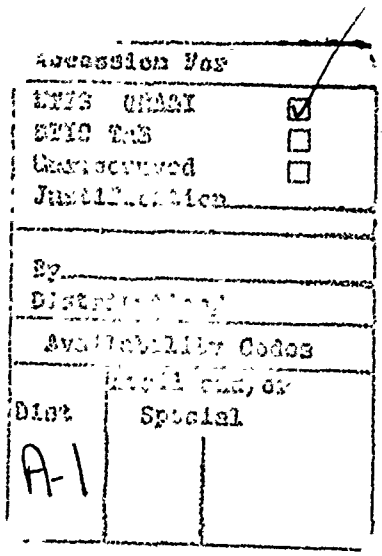

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ACKNOWLEDGEMENT

The authors wish to acknowledge the kind assistance of Ms. Maureen Simpson and Dr. Bill Kournikakis in the evaluation of the *in vitro* neutralizing activity of the antibody.

ABSTRACT

Liposome-mediated passive immunity was evaluated for its efficacy in the prophylaxis and treatment of influenza A/PR/8 virus infection in mice. Avirulent, egg-propagated influenza A/PR/8 virus (H1N1) was adapted for growth in Balb/c mice. In the *in vivo* protection study, purified polyclonal antibody (PA) which demonstrated strong reactivity against the mouse-adapted virus in an indirect fluorogenic enzyme-linked immunosorbent assay (FELISA) and *in vitro* plaque assay, was encapsulated within liposomes. Using I^{125} – IgG as a radioactive tracer for the antibody molecules, the delivery of antibody to the lungs was optimized by intranasal administration of PA encapsulated within negatively charged multilamellar vesicles made from phosphatidylcholine: cholesterol: phosphatidylserine (7: 2: 1). For mice given PA intranasally 24 hours prior to challenge with 10 LD_{50} of mouse-adapted influenza A/PR/8 virus, the survival rate at 14 days post challenge was 60% ($P < 0.05$), compared to 0% for the control groups of mice given either phosphate-buffered saline (PBS) or sham liposomes. However, when mice were given PA encapsulated within liposomes (LIP-PA), the survival rate was increased significantly from 60% to 100% ($P < 0.05$). In the treatment of mice already infected with 10 LD_{50} of the virus, mice which were given PA or LIP-PA were fully protected (100% survival rate), provided that the mice were treated within 8 hr post infection with PA, or within 12 hr with LIP-PA. These results suggest that passive immunity was efficient in the prophylaxis and treatment of influenza A/PR/8 infection in mice and that its efficacy can be further enhanced when liposomes were used as carriers for PA.

RÉSUMÉ

L'efficacité de l'immunité passive à méditation liposomique dans la prophylaxie et le traitement du virus de la grippe A/PR/8 a été étudiée chez la souris. Le virus de la grippe A/PR/8 (H1N1) avirulent et propagé sur oeuf a été adapté pour croî tre dans les souris Balb/c. Dans l'étude de protection *in vivo*, un anticorps polyclonal purifié (APP) ayant montré une forte réactivité avec le virus adapté aux souris lors d'un test FELISA à immunofluorescence indirecte (FELISA) ainsi qu'avec la méthode *in vitro* des plaques de lyse, a été encapsulé dans des liposomes. On a utilisé l'IgG marquée à l'iode^{125} comme traceur radioactif pour les molécules d'anticorps et on a optimisé la dose reçue par les poumons en ayant recours à l'administration par voie intranasale d'APP encapsulé dans des vésicules multilamellaires électronégatives formées de phosphatidylcholine, de cholestérol et de phosphatidylsérine dans un rapport 7: 2: 1. Chez les souris injectées avec l'APP par voie intranasale 24 heures avant l'administration de 10 DL_{50} du virus de la grippe A/PR/8 adapté aux souris, le taux de survie 14 jours après l'infection a été de 60% ($P < 0,05$). Le taux de survie des souris du groupe-témoin ayant reçu une solution tamponnée au phosphate ou des liposomes sans APP a été de 0%. Le taux de survie s'est accru de façon significative, de 60 à 100% ($P < 0,05$), chez les souris ayant reçu l'APP encapsulé dans des liposomes (APP-LIP). Les souris déjà infectées avec 10 DL_{50} de virus ont pu être protégées à 100% par l'administration d'APP ou d'APP-LIP dans un délai n'excédant pas 8 heures (12 heures pour l'APP-LIP) après l'infection. Les résultats laissent entendre que l'immunité passive a été efficace dans la prophylaxie et le traitement du virus de la grippe A/PR/8 chez les souris et que l'utilisation de liposomes comme porteurs de l'APP augmenterait cette efficacité.

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INTRODUCTION

Influenza is a major disease of humans. It is estimated that the great influenza pandemic of 1918-1920 killed more than 20 million persons and was one of the most devastating plagues in human history. Influenza virus infection and subsequent complications from secondary bacterial pneumonia can cause death in the elderly and in immunologically compromised persons, and can cause prolonged incapacitation in healthy individuals. As a result of the high human mortality and morbidity rate influenza can inflict, influenza viruses are considered as potential BW threat agents.

Influenza viruses are members of the orthomyxoviridae. They are relatively large enveloped viruses whose genome contains single-stranded RNA which is divided and segmented (1). Human influenza is normally transmitted by the aerosol route and pathogenesis is characterized by upper respiratory tract infection which may develop into a more severe lower respiratory pneumonitis, and which can be further be complicated with a secondary bacterial infection (2). Vaccination against influenza is difficult because of the remarkable ability of the influenza viruses to change their antigenic structure by frequent antigenic drifts and shifts in the genes coding for the virus spike proteins neuraminidase and hemagglutinin (3, 4). Immunity against influenza infection is induced by the hemagglutinin protein and can be evoked by injection with purified hemagglutinin (5). Recently, passive transfer of hemagglutinin-specific antibody has been shown to protect mice from infection with lethal doses of influenza A virus (6). However, protection with this antibody was only 60% effective, and was completely ineffective in the treatment of mice already infected with the

virus. It is possible that the antibody administered was not protected from in vivo dilution and degradation, or could not reach the cellular sites of infection. Liposomes, which are microscopic lipid vesicles, provide an attractive antiviral delivery system as they can be targeted to deliver the antiviral agents to the organ or cellular sites of infection. In addition, liposome-encapsulated antiviral agents are protected from in vivo dilution and degradation, and are released in a gradual and sustained manner. As a result, the therapeutic and prophylactic indexes of these antimicrobial agents can be dramatically improved while their inherent toxicity is significantly reduced. In this study, liposomes were evaluated for their effectiveness as delivery system for the targeting of purified polyclonal antibody (PA) to the lower respiratory tract (i.e. the lungs). The respiratory system is the most common route of entry for airborne pathogens, whether during BW attack or in natural disease transmission. In this study, influenza A/PR/8 virus, passaged and adapted in mice, was used as a virus model system. We evaluated the efficacy of liposome-encapsulated antibody (LIP-PA) for the prophylaxis and therapy of influenza virus infection in mice.

MATERIALS AND METHODS

Animals

Female Balb/c mice, aged 5-6 weeks, were purchased from Charles River Ltd. (St. Constant, PQ). Upon the arrival at DRES, the mice were quarantined for a week in the vivarium, and were housed and cared for in a manner consistent with the guidelines set by the Canadian Council on Animal Care.

Reagents

All phospholipids and cholesterol used for the preparation of liposomes in this study were purchased from Sigma Chemical Company (St. Louis, MO). Goat IgG labelled with ^{125}I (specific activity, 2-15 uCi/ug) was obtained from ICN Biochemicals Canada Ltd. (Montreal, PQ). Affinity purified alkaline phosphatase-labelled rabbit anti-goat IgG was from Sigma Chemical Company. Purified goat antibody directed against influenza A virus strains A-USSR (H1N1) and Victoria (H3N2) was obtained from Bio/Can Scientific Inc. (Mississauga, Ont.).

Adaptation of egg-propagated influenza A/PR/8 virus in mice

Influenza A/PR/8 virus was adapted in mice by four blind passages, using egg-propagated virus (ATCC, Parklawn, Md.) as the initial inoculum. For each passage, Balb/c mice, anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.), were inoculated intranasally with 50 ul of egg-propagated virus for the initial passage. At four days post infection, the mice were sacrificed and the lungs were aseptically removed. The lungs were then grounded in a tissue grinder with a mixture of sterile aluminium hydroxide powder (5 g) and phosphate-buffered saline (PBS, pH 7.2, 10 ml) containing penicillin-G (100 ug/ml), fungizone (0.25 ug/ml) and streptomycin sulfate (100 ug/ml). The ground lung extract was then centrifuged at 5,000 x g for 15 min and the supernatant was used for re-inoculation into mice in subsequent passages. The supernatant from the fourth and final passage was inoculated into the allantoic cavity of embryonated hens' eggs and the eggs were incubated at 37^{o}C for 4-5 days. The allantoic fluids were then isolated and pooled. The pooled allantoic fluid was assayed by hemagglutination (HA) and by a mouse LD_{50} assay.

HA assay

HA assays were performed with 0.5% rooster erythrocytes (Institute Armand Frappier, Laval, PQ) by a standard technique (7).

LD_{50} determination for mouse-adapted influenza A virus

Pooled allantoic fluid from embryonated eggs infected with lung extract from the fourth passage in mice was diluted serially in sterile PBS. Balb/c mice, anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), were inoculated intranasally with 50 ul of the virus dilutions (8 mice per group). At day 14 post infection, the number of mice which had survived the virus infection was recorded. The LD_{50} value was calculated by the method of Reed and Muench (8).

Plaque and plaque inhibition assays

The plaque assay used for the titration of the mouse-adapted influenza A/PR/8 virus was essentially as described for Newcastle disease virus (9) with the exception that MDCK cells (American Type Culture Collection, Rockville, MD) were used in place of $LLC\text{-}MK_2$ cells. For the plaque inhibition assay, purified antibody directed against influenza A/PR/8, diluted 10-fold serially in PBS, was mixed and coincubated with influenza virus at $37^{\circ}C$ for 30 min before being titrated by the plaque assay as described above.

Indirect fluorogenic enzyme-linked immunosorbent assay (FELISA)

The immunoreactivity of the purified PA for the mouse-adapted influenza A virus was determined by an indirect FELISA. Wells of Millititer^{TM} HA plates were coated with mouse-adapted virus, by adding to the wells 50 ul of allantoic fluid (diluted 1 : 1,000 in 0.05 M carbonate-bicarbonate buffer, pH 9.6), followed by overnight incubation of the plate at 4^{O}C. PA, diluted serially in PBS containing 2% bovine serum albumin and 0.5% Tween 20, was added to the wells. All other steps were essentially as described before (10), with the exception that the antibody-enzyme conjugate used was alkaline phosphatase-labelled rabbit anti-goat IgG.

Liposome preparation

Liposomes used for the encapsulation of antibody were prepared by a modification of the freeze drying method of Kirby and Gregoriadis (11). Negatively charged liposomes were prepared from phosphatidylcholine : cholesterol : phosphatidylserine at a molar ratio of 7 : 2 : 1, and positively charged liposomes were prepared from phosphatidylcholine : cholesterol : stearylamine at the same molar ratio. In each case, a total of 20.2 umoles of lipids were used to 200 ul of PA (5 mg/ml). The liposomes were negatively stained with 2% sodium phosphotungstate (pH 7.4) and the morphology and vesicle size distribution was analyzed by electron microscopy. Liposomes prepared using this method were found to be heterogeneous in size with the vesicle diameters ranging from approximately 300 nm to 2 um. The efficiencies of IgG entrapment within liposomes were estimated using goat ^{125}I-IgG as a radioactive tracer of IgG. IgG not associated or

entrapped in liposomes was removed by discarding the supernatant following two cycles of ultracentrifugation at 100,000 x g for 30 min. Efficiency of entrapment of IgG within liposomes was defined as the percentage ratio of radioactive activity associated with the liposome pellet to radioactive count of total ^{125}I-IgG added to the lipid preparation. The efficiencies of IgG entrapment determined using this method were found to be 47% and 45% for negatively charged and positively charged liposomes, respectively.

Liposome and antibody administration

Unless otherwise stated, the volume of inoculum used for intranasal, intratracheal and intravenous administrations of PA, LIP-PA and LIP was 50 ul. For intranasal administration of liposomes or antibody, mice were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.). When the animals were unconscious, they were carefully supported by hands with their nose up, and the material to be administered was gently applied with a micro-pipettor to the inside of one nostril. The applied volume was naturally inhaled into the lungs. For intratracheal administration, the trachea of an anesthetized mouse was surgically exposed and the material to be adminstered was directly injected into the trachea using a 250 ul Hamilton syringe. For intravenous injection, the material was administered by direct injection via the tail vein.

Organ distribution of radioactive tracer

The organ distribution of the radioactive tracer ^{125}I, as an indication of IgG localization, following intravenous, intranasal and intratracheal administrations of ^{125}I-IgG was

evaluated. For each of the three routes of administration, liposomes containing a total of 1 umol total lipid and 0.2 uCi of ^{125}I-IgG was administered to each of the three mice in the group. At 2 hr post administration, the mice were sacrificed by cervical dislocation and spleens, hearts, lungs, liver and approximately 0.5 ml of blood were collected. The radioactive emissions of the organs and of the blood were then measured in a Beckman Gamma 4000 counter (Mississauga, Ont.).

Protection studies by passive immunity

In the study of prophylactic treatment of influenza A infection in mice, groups of sodium pentobarbital-anesthetized mice (10 mice per group) were inoculated intranasally with PA (20 ug per mouse), PA encapsulated within liposomes (LIP-PA, 1 umol total lipid containing 20 ug PA per mouse), or with sham liposomes (liposomes without PA)(LIP, 1 umole total lipid per mouse). At 24 or 48 hr post inoculation, the mice were challenged intranasally with 10 LD_{50} of influenza A/PR/8 virus. At day 14 post virus challenge, the number of mice surviving the virus challenge was recorded.

In the treatment of mice preinfected with 10 LD_{50} of influenza A virus, the infected mice (groups of 4 mice) were, at various time intervals (4, 8, 12, 16, 24 and 48 hr post infection), either treated with PA, LIP-PA or LIP, with the same doses as described above for prophylactic treatment. At day 14 post infection, the number of survivors in each group of mice was recorded.

Statistics

Statistical comparisons of the mortality and survival rates among groups of mice were by analysis of variance (ANOVA), and were calculated using the Multivariate General Linear Hypothesis (MGLH) module of the Systat computer software program (Evanston, IL).

RESULTS

Adaptation of egg-propagated virus in mice

The egg-propagated virus became pathogenic in mice as early as after the second passage. The clinical symptoms observed in the infected mice were standing fur, rapid loss of body weight, grouping together and significant loss of animal's movement inside the cages. Post mortem examination of these mice revealed severe pulmonary lesions and enlargement of the lungs in some of the mice. HA titers of the supernatant from the ground lung extract from the fourth passage in mice and of the allantoic fluid from the final passage in embryonated eggs were found to be 1: 64 and 1: 2,024, respectively.

LD_{50} Determination for mouse-adapted influenza A virus

The LD_{50} determination in mice infected intranasally with mouse-adapted influenza A/PR/8 is shown in Table I. All mice infected with the mouse-adapted virus at a dilution of 10^{-5} or lower died from the infection. The LD_{50} was $10^{-5.21}$. The 50%

survival time, defined as the time by which 50% of the mice died from infection with 10 LD_{50} of the virus, was found to be approximately 7 days.

Titration of antibody activity by plaque inhibition assay

The reactivity of PA towards the mouse-adapted influenza A virus was demonstrated by the ability of PA to neutralize the infectivity of the virus in vitro in the plaque inhibition assay (Fig. 1). PA inhibited virus infectivity in a dose-dependent manner. The neutralizing antibody titer, defined as the recipocal of antibody dilution required to cause a 50% reduction of plaque formation, was extrapolated from the graph and estimated to be approximately 10^4.

Titration of antibody activity by indirect FELISA

The in vitro immunoreactivity of the PA towards the mouse-adapted virus, determined by titration of virus coated on nitrocellulose membrane with varying dilutions of the PA and with optimal dilution of the alkaline phosphatase-labelled rabbit anti-goat IgG, is represented in Fig. 2. The PA was highly reactive towards the mouse-adapted virus in the indirect FELISA, with the antibody titer determined to be 1:100,000.

Optimization of delivery of LIP-PA to the lungs

In order to optimize the delivery of LIP-PA into the lungs, various routes of administration of liposomes (intravenous, intranasal and intratracheal) were evaluated. Liposome-encapsulated ^{125}I-IgG was used as a radiolabelled tracer for an-

tibody molecules. It was found that intranasal administration with negatively charged liposomes was the most efficient method, with as much as 92% of the radioactuve tracer administered being localized in the lungs (Table II). Although intratracheal adminstration was found to be as effective as intranasal administration, the latter method did not require anesthetic nor surgery, and was therefore, less cumbersome to perform. Subsequently, intranasal administration was chosen for routine use in this study. In addition, the IgG delivered to the lungs using negatively charged liposomes was two times as efficiently retained as when positively charged liposomes were used as carriers (Fig. 3). These results indicated that optimum targeting of IgG to the lungs was achieved with intranasal administration using negatively charged liposomes as carriers.

Prophylactic treatment by carrier-mediated passive immunity

In the efficacy evaluation of the PA and LIP-PA for the prophylactic treatment of influenza infection in mice, it was found that PA, when administered to the mice 24 hr prior to challenge with 10 LD_{50} of influenza virus, offered partial (60% survival rate) but significant (F-ratio = 28.5, $P < 0.05$) protection to mice from the virus challenge (Table III). However, when mice were given PA encapsulated within negatively charged liposomes, the survival rate improved significantly from 60% to 100% (F-ratio = 6.0, $P < 0.05$). As expected, mice given sham liposomes were not protected against the virus infection. Mice were fully protected against the virus challenge provided that LIP-PA was administered 24 hr prior to virus challenge. If administered beyond 24 hr prior to virus challenge, the survival rate decreased significantly from 100% at 24 hr to 50% at 48 hr (F-ratio = 8.0, $P < 0.05$).

Treatment of Influenza virus infection

To evaluate the efficacy of PA and LIP-PA for the treatment of influenza A infection, mice infected with 10 LD_{50} of influenza virus were treated, at various time intervals (4, 8, 12, 16, 48 hr post infection), with either PA, LIP-PA or sham liposomes. Treatments with either PA or LIP-PA resulted in highly effective and significant (100% survival rate, $P < 0.000$) protection to that of control mice (Table IV), provided that the mice were treated within 8 hr post infection with PA, or within 12 hr with LIP-PA. All mice in the 4 and 8 hr PA and LIP-PA groups survived the virus challenge. For mice treated with PA, the survival rate decreased significantly when the infected mice were treated after 8 hr post infection. On the other hand, the survival rate in the mice treated with LIP-PA decreased when treated after 12 hr post infection. As expected, all mice in the control groups treated with PBS or sham liposomes died from the virus challenge.

DISCUSSION

Our data which showed that passive transfer of purified PA by the intranasal administration conferred partial prophylactic protection to non-immunized mice *in vivo* is in close agreement with that of McLain and Dimmock (6). In the latter study, McLain and Dimmock demonstrated that transfer of affinity-purified HA specific antibody delivered intranasally to non-immunized mice 24 hr before challenge with 4 LD_{50} of influenza A/WSN (H1N1) virus offered 60% survival rate in the infected mice. However, there is an apparent discrepancy between the two studies in that while McLain and Dimmock found passively transferred antibody was

completely ineffective in the treatment of mice lethally infected with the virus, we found the infected mice can be effectively treated (100% survival rate) with intranasal administration of the antibody, provided that the antibody is administered within 8 hr after virus challenge. The reason for this discrepancy is likely attributable to the fact that in their study, the infected mice were treated with the antibody at 24 hr post infection at which time the virus infection may have spread beyond the lung, thereby avoiding effective treatment.

The mechanism by which passive transfer of antibody confer in vivo protection to non-immunized mice is not entirely understood but may be associated with several antibody-virus interactions. The antibody molecules could directly neutralize the infecting virus, thus preventing the adsorption and penetration of the virus particles into the host cells in the lungs. Viruses such as influenza which spread extracellularly from the infected cells to the extracellular milieu are particularly susceptible to antibody neutralization (12). In addition, the coating of the extracellular virus by the antibody molecules may facilitate the phagocytosis and intracellular destruction of virus by pulmonary macrophages and polymorphonuclear leukocytes. The binding of the IgG molecules to the virus particles could also activate the cytolytic destruction of the virus particles by the complement pathway. However, infection with influenza virus can also result in significant decrease in the phagocytic capability of the pulmonary macrophages (13, 14), an effect which could partly account for the high rate of secondary bacterial pneumonia in human influenza infection (2), and may partly explain the relatively short period after virus infection in which treatment must be administered for it to be effective.

Encapsulation of the antibody molecules within negatively-charged liposomes significantly increased the survival rate from 60% to 100% in mice which were given LIP-PA 24 hr prior to virus challenge. This improvement of protection against influenza infection was probably not due to the non-specific activation of the pulmonary phagocytic macrophages by liposomes since sham liposomes, administered to mice in the control group, did not confer protection against the virus challenge. Our radiotracer study using ^{125}I-IgG indicated than more than 45% of the radioactive tracer delivered in liposomes was retained in the lungs after 24 hr post administration, compared to 10-15% for the free IgG, suggesting that the improved survival rate was likely a direct result of better IgG retention in the lungs when liposomes were used as carriers. It is not known why the antibody was better retained in the lungs when negatively-charged liposomes containing phosphatidylserine, rather than positively-charged liposomes, were used as carriers for PA. This observation is consistent with the findings of Fidler et al., (15) and may suggest that there is a specific interaction between negatively-charged liposomes with the lung endothelial cells. The observation that the rate of IgG retention in the lungs in this study is consistent with the reported rate of retention for liposomes in the adult rabbit lungs (16) suggests that the IgG retained may still be associated with intact liposomes. Our data, along with that of others (16, 17), indicate that the liposome-encapsulated materials were likely to be protected from dilution and *in vivo* degradation in the lungs. The fate of liposomes once reaching the lungs remains largely unknown. Liposomes in systemic circulation from intravenous administration do not appear to penetrate the capillary endothelial barrier in the lungs but are trapped in the capillary bed (18). However, there is also strong evidence to support that liposomes reaching

the lungs are rapidly and readily taken up by alveolar macrophages (19, 20), and contents readily released from the liposomes into the lungs (16).

Mice infected with 10 LD_{50} of influenza A virus can be effectively treated (100% survival rate) with LIP-PA, provided that the infected mice are treated within 12 hr post virus challenge. It is likely that treatment of the infected mice by LIP-PA is only effective before the virus infection is established or has spread to other parts of the body, or before the phagocytic capability of the pulmonary macrophages is impaired by the infecting virus.

Injection of foreign proteins into a recipient animal or patient, as in the case of passive immunization, may result in clinical illness. Allergic reaction may range in severity from a local inflammatory reaction to an acute anaphylaxis which may result in cardiovascular collapse, and even death in some cases. However, liposomes may reduce the allergic reaction to these proteins by acting as slow-releasing reservoirs for these antigens.

CONCLUSIONS

Liposome-encapsulation of antibody molecules improved the prophylactic and therapeutic efficacies of passively transferred antibody in protecting mice against intranasal infection with with lethal doses of influenza A virus. This study represents a good model system for liposome targeting and delivery of prophylactics and therapeutics to the lungs. Such targeting and delivery could have important applications in the medical defence against BW agents since the respiratory system is the

most likely route of entry for airborne microorganisms during BW attacks. The potentiation of prophylactic and therapeutic agents by liposomes in this study reinforces the unique potential that application of liposomes has as a delivery system for targeting of antimicrobial agents to specific sites of infection in the body.

REFERENCES

1. Choppin, P.W. and Compans, R.W., "The Structure of Influenza Virus", Kilbourne, E.D. (Ed.), The Influenza Viruses and Influenza, Academic Press, New York, New York, 1975, pp. 15-52.

2. Louria, D.B., Blumenfeld, H.L., Ellis, J.T., Kilbourne, E.D. and Rogers, D.E., "Studies on Influenza in the Pandemic of 1957-1958. II. Pulmonary Complications of Influenza", J. Clin. Invest., 38 (1959) pp. 213-265.

3. Lamb, R.A. and Choppin, P.W., "The Gene Structure and Replication of Influenza Virus", Ann. Rev. Bioch., 52 (1983) pp. 467-506.

4. Murphy, B.R. and Webster, R.G., "Influenza Viruses", Fields, B.N., Chanock R.M., Melnick, J.L., Roizman, B. and Shope, R.E. (Eds.), Virology, Raven Press, New York, 1985, pp. 1179-1239.

5. Ginsberg, H.S., "Orthomyxoviruses", Davis, B.D., Dulbecco, R., Eisen, H.N. and Ginsberg, H.S., (Eds.), Microbiology, Harper and Row, Publishers, Inc., Philadelphia, Pennsylvania, 1980, pp. 1120-1138.

6. McLain, L. and Dimmock, N.J., "Protection of Mice from

Lethal Influenza by Adoptive Transfer of Non-neutralizing Haemagglutination-inhibiting IgG Obtained from Lungs of Infected Animals Treated with Defective Interfering Virus", J. Gen. Virol., 70 (1989) pp. 2615-2624.

7. Grist, N.R., Ross, C.A. and Bell, E.J., "Hemagglutination and Hemagglutination Inhibition Assays", Diagnostic Methods in Clinical Virology, Blackwell Scientific Publications, London, England, 1974, pp. 103-111.

8. Dulbecco, R. and Ginsberg, H. S., "The Nature of Viruses", Davis, B.D., Dulbecco, R., Eisen, H.N. and Ginsberg, H.S. (Eds.), Microbiology, Harper and Row, Publishers, Phildelphia, Pennsylvania, 1980, pp. 854-884.

9. Kournikakis, B. and Fildes, J., "Titration of Avirulent Newcastle Disease Virus by Plaque Assay Method", J. Virol. Methods, 20 (1988) pp. 285-293.

10. Fulton, R.E., Wong, J.P., Siddiqui, Y.M. and Tso, M.-S., "Sensitive Flucrogenic Enzyme Immunoassay on Nitrocellulose Membranes for Quantitation of Virus", J. Virol. Methods, 22 (1988) pp. 149-164.

11. Kirby, J.K. and Gregoriadis, G., "A Simple Procedure for Preparing Liposomes Capable of High Efficiency Under Mild Conditions", Gregoriadis, G. (Ed.), Liposome Technology, CRC Press, Inc., Boca Raton, Florida, 1983, pp. 19-27.

12. Drutz, D.J. and Mills, J., "Immunity and Infection", Stites, D.P., Stobo, J.D., Fudenberg, H.H. and Wells, J.V. (Eds.), Basic and Clinical Immunology, Lange, Los Altos, Calif., 1982, pp. 209-232.

13. Jakab, G.J., Warr, G.A. and Knight, M.E., "Pulmonary and Systemic Defences against Staphylococcus aureus in Mice with Pneumonia Due to Influenza A Virus", J. Infec. Dis., 140 (1979) pp. 105-108.

14. Warhauer, D., Goldstein, E., Akers, T., Lippert, W. and Kim, M., "Effect of Influenza Viral Infection on the Ingestion and Killing of Bacteria by Alveolar Macrophages", Am. Rev. Respir. Dis., 115 (1977) pp. 269-277.

15. Fidler, I.J., Hart, I.R., Raz, A., Fogler, W.E., Kirsh, R. and Poste, G., "Activation of Tumoricidal Properties in Macrophages by Liposome-encapsulated Lymphokines: In Vivo Studies", Tom, B.H. and Six, H.R. (Eds.), Liposomes and Immunobiology, Elsevier, N.Y., 1980, pp. 109-117.

16. Pettenazzo, A., Jobe, A., Ikegami, M., Abra, R., Hogue, E. and Mihalko, P., "Clearance of Phosphatidylcholine and Cholesterol from Liposomes, Liposomes Loaded with Metaproterenol, and Rabbit Surfactant from Adult Rabbit Lungs", Am. Rev. of Respir. Dis., 139 (1989) pp. 752-758.

17. Debs, R.J., Straubinger, R.M., Brunette, E.N., Lin, J.M.,

Lin, E.J., Montgometry, A.B., Friend, D.S. and Papahadjopoulos, D.P., "Selective Enhancement of Pentamidine Uptake in the Lung by Aerosolization and Delivery in Liposomes", Amer. Rev. of Respir. Dis., 135 (1987) pp. 731-738.

18. Hunt, C.A., Rustum, Y.M., Mayhew, E. and Papahadjopoulos, D., "Retention of Cytosine Arabinoside in Mouse Lung Following Intravenous Administration in Liposomes of Different Size", Drug Metab. Dispos., 7 (1979) pp 124-128.

19. Fidler, I.J., Raz, A., Fogler, W.E., Kirsch, R., Bugelski, P., and Poste, G., "Design of Liposomes to Improve Delivery of Macrophage Augmenting Agents to Alveolar Macrophages", Cancer Res., 40 (1980) pp. 4460-4466.

20. Post, G., Kirsch, R., Raz, A., Sone., S., Bucana, C., Fogler, W.E. and Fidler, I.J., "Activation of Tumoricidal Properties in Macrophages by Liposome Encapsulated Lymphokines: In Vitro Studies", Tom, B.H. and Six, H.R. (Eds.), Liposomes and Immunobiology, Elsevier, N.Y., 1983, pp. 93-107.

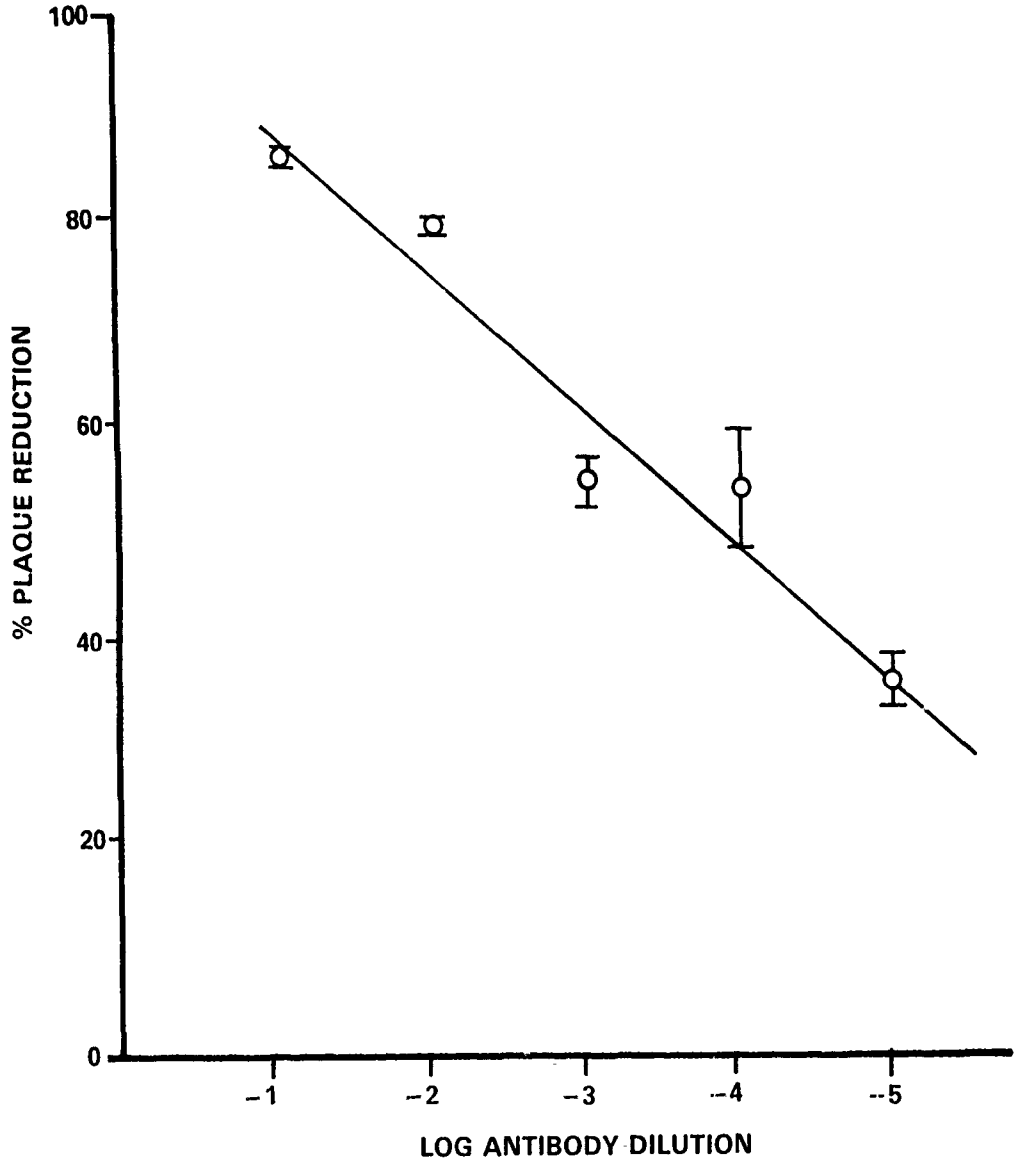

Figure 1

IN VITRO **NEUTRALIZATION ACTIVITY OF PA DETERMINED BY PLAQUE INHIBITION ASSAY. PA, diluted serially in sterile PBS, was mixed with an appropriate concentration of the mouse-adapted virus. Following incubation at 37°C for 30 min, the virus-antibody mixture were titrated by the plaque assay. Data points represent the mean of triplicate determinations. Error bars shown are standard deviations of the means.**

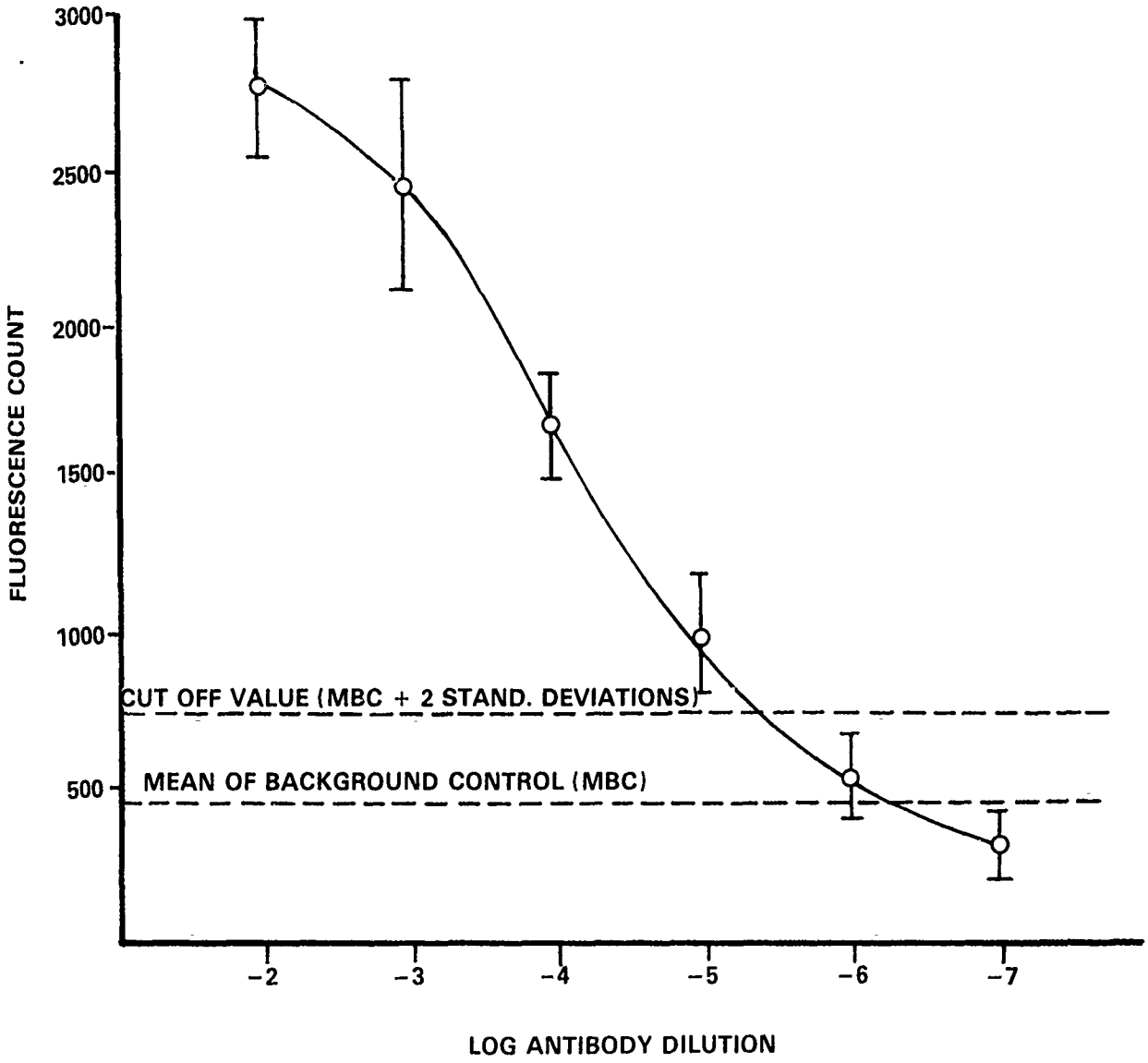

Figure 2

IMMUNOREACTIVITY OF PA DETERMINED BY "INDIRECT" FELISA. Varying dilutions of PA (10^{-2} to 10^{-7}) were titrated by "indirect" FELISA and fluorescence counts were determined. Data points represent the mean of triplicate determinations on a single plate. Error bars represent the standard deviations of the means.

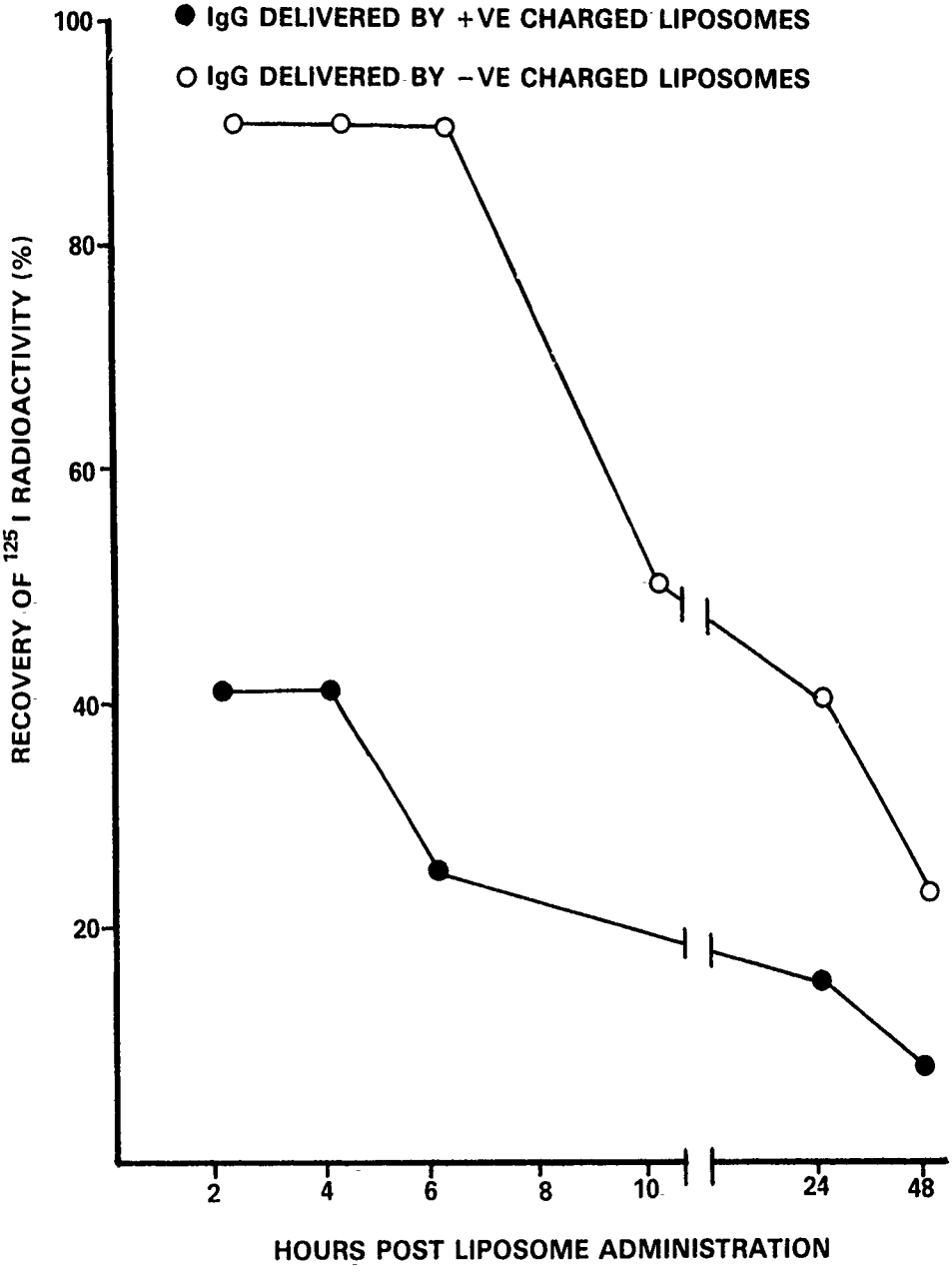

Figure 3

COMPARISON OF LUNG RETENTION OF IgG WHEN POSITIVELY AND NEGATIVELY CHARGED LIPOSOMES WERE USED AS CARRIERS FOR IgG. Positively and negatively charged liposomes (1 μmole total lipid) containing 0.2 μCi ^{125}I-IgG were administered intranasally to mice. At various time intervals, the mice were sacrificed and the lungs were removed. The radioactive emissions of the lungs were then measured in a Beckman Gamma 4000 counter. Data points represent the means of duplicate determinations.

Table I

LD_{50} DETERMINATION IN BALB/C MICE INFECTED INTRANASALLY WITH MOUSE-ADAPTED INFLUENZA A VIRUS. Dilutions of allantoic fluid (10^{-4} to 10^{-7} in PBS) from embryonated eggs infected with lung extract from the fourth passage in mice were administered intranasally to groups of Balb/c mice (50 μl per mouse). At day 14 post infection, the number of mice which had died from the infection was recorded.

LOG OF VIRUS DILUTION	NO. SURVIVORS AT DAY 14 POST CHALLENGE	% MORTALITY
−4	0/8	100
−5	0/8	100
−6	5/8	37
−7	8/8	0

Table II

ORGAN AND BLOOD DISTRIBUTION OF RADIOACTIVE TRACER ^{125}I AT 2 hr FOLLOWING INTRAVENOUS, INTRANASAL AND INTRATRACHEAL ADMINISTRATIONS OF ^{125}I-IgG DELIVERED BY NEGATIVELY CHARGED LIPOSOMES. For each of the three routes of administration, liposomes at 1 μmole total lipid containing 0.2 μCi ^{125}I-IgG was administrated to groups of three mice. At 2 hr post administration, the mice were sacrified by cervical dislocation and their spleens, lungs, hearts, livers and approximately 0.5 ml of the blood were collected. The radioactive emissions of the organs and of the blood were then measured in a Beckman Gamma 4000 counter. * denotes significant difference ($p < 0.05$) from % radioactive marker recovery in lungs of mice following intravenous injection.

	INTRANASAL	INTRAVENOUS	INTRATRACHEAL
LUNG	92%* ± 9%	14% ± 3%	90%* ± 6%
SPLEEN	–	9% ± 4%	–
LIVER	2% ± 1%	55% ± 5%	2% ± 1%
STOMACH	5% ± 2%	–	6% ± 3%
BLOOD (0.5 ml)	–	3% ± 2%	–

Table III

PROPHYLACTIC TREATMENT OF MICE INFECTED WITH MOUSE-ADAPTED INFLUENZA A VIRUS. Mice anesthetized with sodium pentobarbital (50 mg/kg body weight) were inoculated intranasally (50 μl) with PA (20 μg per mouse), LIP-PA (1 μmole total lipid containing 20 μg PA), sham liposomes (1 μmole total lipid), or PBS. At 24 or 48 post administration, the mice were infected intranasally with 10 LD_{50} of mouse-adapted influenza A virus. At day 14 post infection, the number of mice surviving the infection was recorded.

GROUP	HOURS PRIOR TO VIRUS CHALLENGE	NO. OF SURVIVORS DAY 14 POST CHALLENGE	% SURVIVORS
PBS	24, 48	0/20	0
FREE PA	24	6/10	60
FREE PA	48	4/10	40
LIP-PA	24	10/10	100
LIP-PA	48	5/10	50
SHAM-LIP	24, 48	0/20	0

Table IV

TREATMENT OF MICE INFECTED INTRANASALLY WITH 10 LD_{50} OF MOUSE-ADAPTED INFLUENZA A VIRUS. Groups of mice preinfected with 10 LD_{50} of the influenza A virus were treated at various time intervals post infection (4, 8, 12, 16, 24, and 48 hr), with PA, LIP-PA, sham liposomes, or PBS and the number of survivors was recorded as previously described.

GROUP	HOURS POST VIRUS CHALLENGE	NO. SURVIVORS DAY DAY 14 POST CHALLENGE	% SURVIVORS
PBS	4, 8, 12, 16	0/16	0
	4	4/4	100
	8	4/4	100
FREE PA	12	1/4	25
	16	1/4	25
	48	1/4	25
	4	4/4	100
	8	4/4	100
LIP-PA	12	4/4	100
	16	2/4	50
	48	1/4	25
SHAM LIP	8, 16	0/8	0

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Liposome-mediated passive immunity was evaluated for its efficacy in the prophylaxis and treatment of influenza A/PR/8 virus infection in mice. Avirulent, egg-propagated influenza A/PR/8 virus (H1N1) was adapted for growth in Balb/C mice. In the in vivo protection study, purified polyclonal antibody (PA) which demonstrated strong reactivity against the mouse-adapted virus in an indirect fluorogenic enzyme-linked immunosorbent assay (FELISA) and in an in vitro plaque assay, was encapsulated within liposomes. Using I^{125}-IgG as a radioactive tracer for the antibody molecules, the delivery of antibody to the lungs was optimized by intranasal administration of PA encapsulated within negatively charged multilamellar vesicles made from phosphatidylcholine:cholesterol:phosphatidylserine (7:2:1). For mice given PA intranasally 24 hours prior to challenge with 10 LD_{50} of mouse-adapted influenza A/PR/8 virus, the survival rate at 14 days post challenge was 60% ($P < 0.05$), compared to 0% for the control groups of mice given either phosphate-buffered saline (PBS) or sham liposomes. However, when mice were given PA encapsulated within liposomes (LIP-PA), the survival rate was increased significantly from 60% to 100% ($P < 0.05$). In the treatment of mice already infected with 10 LD_{50} of the virus, mice which were given PA or LIP-PA were fully protected (100% survival rate), provided that the mice were treated within 8 hr post infection with PA, or within 12 hr with LIP-PA. These results suggest that passive immunity was efficient in the prophylaxis and treatment of influenza A/PR/8 infection in mice and that its efficacy can be further enhanced when liposomes were used as carriers for PA.

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